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Occurrence of *Ehrlichia canis* and *Anaplasma platys* in household dogs from northern Parana

Ocorrência de *Ehrlichia canis* e *Anaplasma platys* em cães domiciliados da região norte do Paraná

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Abstract

Canine monocytic ehrlichiosis caused primarily by *Ehrlichia canis* and canine thrombocytic anaplasmosis induced by *Anaplasma platys* are important emerging zoonotic tick-borne diseases of dogs. There is evidence that these pathogens can also affect humans. This study evaluated the presence of *E. canis* and *A. platys* in blood samples collected from 256 domiciled dogs in the municipality of Jataizinho, located in north region of the State of Parana, Brazil, by PCR assay. The occurrence of *E. canis* and *A. platys* was 16.4% (42/256) and 19.4% (49/256), respectively; while 5.47% (14/256) of the dogs evaluated were co-infected by these two organisms. The presence of *E. canis* and *A. platys* was not significantly associated with the variables evaluated (sex, age, outdoor access, and presence of ticks during blood collection). Infection of dogs by *E. canis* was associated with anemia and thrombocytopenia, while infection induced by *A. platys* was related only to thrombocytopenia. Canine monocytic ehrlichiosis and canine thrombocytic anaplasmosis should be included in the differential diagnoses when these hematological alterations are observed during routine laboratory evaluation of dogs.

Keywords: Canine monocytic ehrlichiosis, canine thrombocytic anaplasmosis, PCR, anemia, thrombocytopenia.

Resumo

Erliquiose monocítica canina, causada principalmente por *Ehrlichia canis*, e anaplasmose trombocítica canina, devida a infecção com *Anaplasma platys*, são importantes doenças transmitidas por carrapatos que acometem os cães, com evidências que podem também acometer o homem. O presente estudo avaliou a ocorrência desses agentes em amostras de sangue de 256 cães domiciliados na cidade de Jataizinho, na região Norte do Paraná, Brasil, utilizando a técnica da Reação em Cadeia da Polimerase (PCR). A ocorrência de *E. canis* e *A. platys* foi de 16,4% (42/256) e 19,4% (49/256), respectivamente, com 5,47% (14/256) dos animais apresentando coinfecção. Não foi observada associação significativa com as variáveis sexo, idade, acesso à rua e presença de carrapatos no momento da coleta de sangue. A infecção por *E. canis* teve relação com anemia e com trombocitopenia, enquanto a infecção por *A. platys* apresentou relação apenas com trombocitopenia. Com base nos resultados obtidos, reforçou-se a necessidade de que erliquiose e anaplasmose canina devem estar entre os diagnósticos diferenciais, quando da detecção de anemia e trombocitopenia em exames laboratoriais.

Palavras-chave: Erliquiose monocítica canina, anaplasmose trombocítica canina, PCR, anemia, trombocitopenia.

Introduction

Canine monocytic ehrlichiosis (CME) and canine thrombocytic anaplasmosis (CTA) are infectious diseases caused by gram-negative bacteria of the Order Rickettsiales, family Anaplasmataceae, genera *Ehrlichia* and *Anaplasma* (DUMLER et al., 2001). *Ehrlichia canis* and *Anaplasma platys* are obligatorily intracellular organisms organized in clusters, called morulae, and frequently observed in leukocytes and platelets, respectively, with the possibility of concomitant infections (McBRIDE et al., 1996; COHN, 2003; SUKSAWAT et al., 2001a). These infectious diseases are of great importance for small animal clinics and public health, since they are increasingly prevalent in dogs and because there is evidence that these pathogens can also affect humans (DAGNONE et al., 2001; TAMÍ; TAMÍ-MAURY, 2004; NEER; HARRUS, 2006).

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The transmission of *E. canis* occurs primarily through the bite of the tick, *Rhipicephalus sanguineus*, and this tick might be associated with the transmission of *A. platys* to dogs (INOKUMA et al., 2000; SUKSAWAT et al., 2001b). Since there is a common vector for both diseases, coinfections in animals are frequent (KORDICK et al., 1999; YABSLEY et al., 2008; DAGNONE et al., 2009).

Routine diagnoses of CME and CTA are based on characteristic clinical and hematological findings. The identification of hemoparasites in blood smears is the most widely used technique in clinical practice to characterize morulae in leukocytes and platelets, but this method has low diagnostic sensitivity and specificity and must be supplemented with the use of molecular techniques, such as PCR (NAKAGHI et al., 2008; DAGNONE et al., 2009; RAMOS et al., 2010).

Existing data of CME and CTA within the region of Londrina, north region of the State of Parana, is restricted to hospital cases (DAGNONE et al., 2003; TRAPP et al., 2006); no study evaluating the occurrence of these diseases in household dogs has been found. This study evaluated the occurrence of *E. canis* and *A. platys* in a population of apparently healthy household dogs within the region of Londrina, associating the presence of these agents with possible risk factors and hematological alterations.

Materials and Methods

1. Animal, samples, and study area

Blood samples with EDTA from 256 household dogs were collected from July to August 2010 in the municipality of Jataizinho, located in the north region of the State of Parana, Brazil. These animals, whose owners agreed to participate in this study, were gathered from 124 residences. The number of dogs selected was determined by Epi 6.0 with an expected prevalence of 50%, 5% of precision, and 95% of confidence level.

Part of each sample was kept frozen at –20 °C until DNA extraction, and the remainder was used for hematological analysis. Blood sampling was approved by the Ethics Committee for Animal Experiments (# 34/2011) of the Universidade Estadual de Londrina, Parana state, Brazil., "Universidade Estadual de Londrina" – UEL.

The city of Jataizinho is sidelined by the Tibagi River; it is close to the Tropic of Capricorn, 352 m above sea level, located at coordinates 23° 15' S and 50° 58' W. The climate is humid subtropical with hot summers, classified as Cfa according to the Köppen classification. The average annual temperature in Jataizinho is 21.3 °C, with one rainy season, high temperatures during the spring and summer, being cold and dry during the fall and winter (IAPAR, 2011).

2. Hematological evaluations

The blood samples collected from the dogs were centrifuged to determine the packed cell volume (PCV) by the microhematocrit method (THRALL, 2007). Duplicate blood smears were fixed with methanol, dried at room temperature and stained with Giemsa to estimate the number of platelets per slide (SILVA et al., 2007). Animals were considered anemic when PCV was lower than 37%; they were considered thrombocytopenic when platelet count was smaller than 120,000.

3. DNA extraction and PCR

Genomic DNA was extracted from all blood samples with the use of QIAmp DNA Blood Mini Kit (Qiagen™, Sao Paulo, Brazil), and used for PCR analyses. Molecular-grade water was extracted to confirm that no cross-contamination between samples occurred during DNA extraction.

The primers (EcavB9of, 5'-CATTATCATT-TCAATACGTAACTC-3'; EcavB9or, 5'-TTTTGATT-TTCTTCTGACATAGTG-3') were used to amplify 959 base pairs (bp) of the virB9 gene of *E. canis* from genomic DNA (FELEK et al., 2003). The 504 bp fragment of 16S rRNA gene of *A. platys* was amplified by using the primers platys 16S F (5'-AAGTCGAACGGATTTTTGTC-3'), and platys 16S R (5'-CTCTCCCGGACTCTAGTC-3') (GOTSCH et al., 2009). According the PCR protocol were used 20 pmol of each primer, 200 µM dNTP, 50 ng of genomic DNA, 1X PCR Platinum buffer, 2mM $MgCl_2$, and 1.25 U Platinum DNA Polymerase^R (Invitrogen Life Technologies, USA) for a final volume of 25 µL. The PCR amplification cycle consisted of 35 cycles of denaturation (94 °C for 1 minute), primer annealing of 1 minute (58 °C for *E. canis*; 60 °C for *A. platys*), and a final extension (72 °C for 7 minutes). The PCR products were separated by electrophoresis in 1.5% agarose gel stained with SyBr Safe (Invitrogen™, USA) and visualized under UV light. The 100-pb ladder (Promega, Madison, USA) was used as standard for determining the molecular mass of PCR products. Positive controls consisted of *E. canis* and *A. platys* DNA extracted from the blood of dogs that had positive PCR, and which amplicons were confirmed by sequencing as being from those species, utilizing commercial kit BigDye Terminator (Applied Biosystems, CA, USA); ultra-pure water served as negative control.

4. Statistical analysis

The following variables were analyzed: (a) occurrence of *E. canis* and *A. platys* regarding gender (male and female) of the affected animals; (b) age of infection (0-1 year; 2-5 years; older than 5 years); (c) outdoor access (yes or no); (d) presence of ticks during blood collection (yes or no); and, (e) possible hematological alterations (anemia and thrombocytopenia). Possible associations between the evaluated variables and positive reaction to the agents were determined by the Chi-square test. The probability of error was accepted up to 5% ($p < 0.05$).

Results and Discussion

The results from this study have demonstrated that blood-derived DNA samples of 30.08% (77/256) of the dogs evaluated were positive by PCR assays to at least one infectious agent. However,

10.94% (28/256) of these reacted positively only to *E. canis*, 13.67% (35/256) to *A. platys*, while coinfection was observed in 5.4% (14/256) of these dogs. Nevertheless, the total prevalence (only one agent or coinfection) of *E. canis* and *A. platys* was 16.4% (42/256) and 19.14% (49/256), respectively.

PCR assays successfully amplified the 959 bp of the VirB9 gene of *E. canis* and the 504 bp fragment 16S rRNA gene of *A. platys* from blood-derived DNA samples. The sequencing of amplified DNA from both pathogens showed 99% of identity with known sequences deposited in the GenBank (*E. canis* – AF546158.1 and *A. platys* – GQ395385.1). The prevalence level observed in this study was inferior to those of similar investigations of *E. canis* made in several cities in the country. These included 22% in Londrina, State of Parana, (DAGNONE et al., 2003), 35.6% in Salvador, State of Bahia (SOUZA et al., 2010), and 57% in Recife, State of Pernambuco (RAMOS et al., 2010). Higher prevalence levels were observed in several cities in the State of Sao Paulo: Ribeirao Preto, 38.9% (SANTOS et al., 2009); Jaboticabal [53.3% (NAKAGHI et al., 2008); 72.5% (FARIA et al., 2010); and 88% (DAGNONE et al., 2009)], and Botucatu [30.9% (BULLA et al., 2004); 40% (UENO et al., 2009); and 77.7% (DINIZ et al., 2007)]. Nevertheless, the prevalence level of this study was greater than those described in the city of Rio de Janeiro, State of Rio de Janeiro, 15%, (MACIEIRA et al., 2005) and two cities in the State of Bahia: Ilheus (10.7%; 9/84) and Itabuna (4.3%; 3/69) (CARVALHO et al., 2008). It must be emphasized that most of these studies were performed with dogs attended at Veterinary Hospitals, or with clinical manifestations and/or hematological alterations consistent with CME. Alternatively, the animals from this study were apparently healthy and the samples were collected during the winter, when the environmental population of ticks in that region is comparatively reduced relative to the spring and summer. Taken together, these two conditions might have influenced the lower rates of infection by *E. canis* observed in this study compared to those described elsewhere.

When the result (19.4%) of *A. platys* was compared with similar studies, it was possible to observe that the prevalence level derived from this study was lower than that described in Campo Grande, State of Mato Grosso do Sul, 42% (DAGNONE et al., 2009), and Recife, State of Pernanbuco, 55% (RAMOS et al., 2009). However, these results were higher than those observed in Rio de Janeiro, State of Rio de Janeiro, 15.84% (FERREIRA et al., 2007), Jaboticabal, State of Sao Paulo, 8% (DAGNONE et al., 2009), and Ribeirao Preto, State of Sao Paulo, 14.9% (SANTOS et al., 2009). Moreover, prevalence levels of *A. platys* in the USA, Venezuela, Italy, and Grenada varied from 4% to 55% (KORDICK et al., 1999; HUANG et al., 2005; DE LA FUENTE et al., 2006; YABSLEY et al., 2008). The variation in the percentage of positive animals observed in different studies might be directly related to the canine population evaluated, the degree of exposure to ticks, and the diagnostic method utilized (SOLANO-GALLEGO et al., 2006).

The occurrence (5.47%) of coinfections in dogs caused by *A. platys* and *E. canis* reinforces the hypothesis that these infections are probably transmitted by the same vector; similar results were observed in different geographical regions in Brazil (DANTAS-TORRES, 2008; DAGNONE et al., 2009; RAMOS et al., 2009; SANTOS et al., 2009). This phenomenon was also described in dogs from Thailand (SUKSAWAT et al., 2001b), Venezuela (SUKSAWAT et al., 2001a; HUANG et al., 2005), and Grenada (YABSLEY et al., 2008).

There was no significant association (Table 1) between infection by *E. canis* and the variables evaluated (gender, age, street access, and presence of ticks at the time of blood sampling). Molecular methods demonstrated similar results for the effects of sex and age relative to infection by *E. canis* in Cuiaba, State of Mato Grosso (SILVA et al., 2010), and Ilheus and Itabuna, State of Bahia (CARVALHO et al., 2008). This pattern was also observed with serological assessments performed in several Brazilian cities: Cuiaba, State of Mato Grosso (SILVA et al., 2010), Monte Negro, State of Rondonia (AGUIAR et al., 2007), Patos, State of Paraiba (AZEVEDO et al., 2011), and southern cities in the State of Rio Grande do Sul (SAITO et al., 2008). Serological surveys made in the USA (RODGERS et al., 1989), Israel (HARRUS et al., 1997), and Japan (INOKUMA et al., 1999) revealed similar findings.

Table 1. Risk factors associated with *Ehrlichia canis* in a population of household dogs from Jataizinho, State of Parana, Brazil.

Variables	*N*	PCR (+)	Prevalence (%)	χ^2	*p*-value
Gender					
Male	142	26	18.3	0.472	0.4921
Female	112	16	14.28		
Age					
<1 year	43	6	13.95	0.6291	0.8897
1 to 5 years	148	24	16.22		
>5 years	57	11	19.29		
Not determined	8	1	12.5		
Street access					
Yes	143	22	15.38	0.1067	0.744
No	113	20	17.70		
Presence of ticks at sampling					
Yes	29	5	17.24	0.01885	0.8908
No	227	37	16.3		

N, number of dogs; +, number of dogs positive by PCR.

Alternatively, previous contact of dogs with ticks increased the risk of development CME (TRAPP et al., 2006).

Nevertheless, a higher prevalence of *E. canis* in adult and aged dogs has been described, and it was related to greater exposition to the vector (WATANABE et al., 2004; RODRIGUEZ-VIVAS et al., 2005; COSTA JUNIOR et al., 2007). Although no relationship was observed between street access and the possibility of infection during this study, dogs from the semi-arid region of the State of Paraiba that had restricted street access were more likely to be infected by *E. canis* due to the greater possibility of contact with infected ticks (AZEVEDO et al., 2011).

Moreira et al. (2003) suggested that dogs exposed to ticks are more likely to present elevated levels of infection by *E. canis*. The absence of significant difference between exposure to ticks and infection in this study might probably be because all samples were collected during the winter. Differences in climatic conditions were attributed as important factors that influence the population dynamics of ticks (COSTA JUNIOR et al., 2007). This was recently demonstrated in a study realized in the State of Minas Gerais, where dogs residing in a geographical location with ideal annual temperature for the development of ticks were shown to be 4.6 times more likely to be seropositive for *E. canis*, when compared to dogs living in cities where the average annual temperature is lower (COSTA JUNIOR et al., 2007). Currently, the only known natural method of transmission is via contact with infected ticks; hence, it is likely that the dogs that reacted positively to *E. canis* might have had contact with the vector prior to the sampling, and ticks might not have been observed during sample collection.

Significant association was not observed (Table 2) between infections induced by *A. platys* and the evaluated variables (sex, age, street access, and presence of ticks at the time of sampling). Different from studies associated with *E. canis*, there is little data regarding the risk factors associated with infections induced by *A. platys* in Brazil (DANTAS-TORRES, 2008).

One of the hematological alterations frequently observed in dogs diagnosed with CME is anemia, which is usually normocytic, normochromic, and nonregenerative, suggesting restricted or no bone marrow response (HARRUS et al., 1997; BULLA et al.; 2004; BORIN et al. 2009; GAUNT et al., 2010). These hematological alterations in this anemic disease are probably caused by the combined or isolated effects of the reactions induced by the monocyte-phagocyte system, cell lysis due to the action of the complement system, and suppression of erythropoiesis in the bone marrow are the mechanisms responsible for the table identified as anemic disease (MOREIRA et al., 2003).

When the number of dogs positive for *E. canis* was evaluated, 28.57% (12/42) were anemic, while only 13.94% (29/208) of dogs that were negative by PCR presented anemia (Table 3). Therefore, the proportion of anemic dogs was significantly higher than that of dogs infected with *E. canis*, suggesting that CME is an important cause of anemia in dogs. Similar findings were described in populations of hospitalized dogs (DAGNONE et al., 2003; NAKAGHI et al., 2008). Alternatively, no positive association

Table 2. Risk factors associated with *Ehrlichia canis* in a population of household dogs from Jataizinho, State of Parana, Brazil.

Variables	*N*	PCR(+)	Prevalence (%)	χ^2	*p*-value
Gender					
Males	142	28	19.72	0.001159	0.9728
Females	112	21	18.75		
Age					
<1 year	43	8	18.6	1.828	0.6088
1 to 5 years	148	27	18.24		
>5 years	57	11	19.3		
Not determined	8	3	37.5		
Street access					
Yes	143	32	22.38	0.09337	0.1867
No	113	17	15.04		
Presence of ticks at sampling					
Yes	29	8	27.58	0.1643	0.3285
No	227	41	18.06		

N, number of dogs; +, number of dogs positive by PCR.

Table 3. Manifestations of anemia and thrombocytopenia relative to *Ehrlichia canis* within a population of household dogs from Jataizinho, State of Parana, Brazil.

Clinical manifestation	PCR positive	PCR negative	χ^2	*p*
Anemia	(*n* = 42)	(*n* = 208)		
Yes	28.57% (12)	13.94% (29)	4.44	0.0311
No	71.43% (30)	86.06% (179)		
Thrombocytopenia	(*n* = 42)	(*n* = 205)		
Yes	59.52% (25)	36.58% (75)	6.69	0.009696
No	40.47% (17)	63.41% (130)		

Table 4. Manifestations of anemia and thrombocytopenia relative to *Anaplasma platys* within a population of household dogs from Jataizinho, State of Parana, Brazil.

Clinical manifestation	PCR positive	PCR negative	χ^2	*p*
Anemia	(*n*= 49)	(*n* = 201)		
Yes	20.41% (10)	15.42% (31)	0.3968	0.5287
No	79.59% (39)	84.57% (170)		
Thrombocytopenia	(*n* = 47)	(*n* = 200)		
Yes	61.70%(29)	35.5% (71)	9.784	0.001761
No	38.29% (18)	64.5% (129)		

between anemia and infection by *E. canis* was observed in dogs from the State of Minas Gerais (COSTA JUNIOR et al., 2007), State of Mato Grosso (SOUZA et al., 2010), and Botucatu, State of Sao Paulo (UENO et al., 2009). These hematological differences might be directly related to the phase of infection at the time of sampling (HARRUS et al., 1997; BULLA et al., 2004), or a manifestation of the diverse pathogenicity of the strain (DAGNONE et al., 2003).

Thrombocytopenia, which is also frequently associated with CME, occurred in 59.52% (25/42) of the dogs evaluated during this study (Table 3), but it was only observed in 36.58% (75/205) of dogs that were PCR-negative for *E. canis*. These results showed that a proportionally significant number of dogs infected by this pathogen were thrombocytopenic, indicating that this hematological alteration is an important clinical manifestation of CME. Similar results confirming the association of thrombocytopenia and infection by *E. canis* in dogs were described in Botucatu, State of Sao Paulo (BULLA et al., 2004) and Rio de Janeiro, State of Rio de Janeiro (MACIEIRA et al., 2005).

Several mechanisms have been proposed to explain the cause of thrombocytopenia in dogs with CME, such as increased consumption of platelets, splenic sequestration, destruction by immune-mediated mechanisms, and associated platelet dysfunction (HARVEY, 2006; GAUNT et al., 2010). However, not all thrombocytopenic dogs from the geographical region where this study was carried out are positive for CME, since it was demonstrated that only 19.7% (12/61) of hospitalized dogs from this area that were infected by *E. canis* were thrombocytopenic (DAGNONE et al., 2003). This would suggest that CME is not the only cause of thrombocytopenia in dogs from this geographical location. A recent study done in the same geographical location suggested that thrombocytopenia was more associated with canine babesiosis, induced by *Babesia vogeli*, relative to CME (TRAPP et al., 2006). Additionally, thrombocytopenia is not observed in all dogs experimentally inoculated with *E. canis* (GAUNT et al., 2010). Further, although this hematological alteration is more frequently observed in cases of CME, thrombocytopenia might also be associated with CTA induced by *A. platys* (SANTOS et al., 2009).

In the present study, no relationship was observed between PCR positivity for *A. platys* and the possibility to develop anemia (Table 4). Similar results were described in an experimental study that did not observe reduction in the packed cell volume of dogs infected with *A. platys* (GAUNT et al., 2010). Most (61.7%; 29/47) dogs that were PCR-positive for *A. platys* presented thrombocytopenia, while only 35.5% (71/200) of dogs that were PCR-negative were thrombocytopenic. Consequently, the proportion of dogs with thrombocytopenia is significantly higher in those infected with *A. platys*, suggesting that canine anaplasmosis is also an important cause of this hematological alteration. Similar associations were described in a recent experimental study (GAUNT et al., 2010).

Different investigative strategies have been used to associate the prevalence of hemoparasites with hematological alterations, including the identification of the infectious agent in dogs with anemia and/or thrombocytopenia. However, this frequently induces bias selection and undermines prevalence data, since thrombocytopenic dogs are more likely to be positive for CME (DAGNONE et al., 2003), while thrombocytopenia is not always associated with CME in some geographical locations of Brazil (TRAPP et al., 2006). Therefore, it would be worth comparing the prevalence of these infectious agents in dogs with and without the characteristic clinical manifestations of CME and CTA. In this case, if prevalence levels are higher in dogs with clinical manifestations, the data should be interpreted as a consequence of the infectious agent and not as risk factors that might predispose the animal to infection (CARLOS et al., 2011).

Alternatively, it is also possible to evaluate dogs that have reacted positively to one infectious agent by comparing the ratio of anemic and/or thrombocytopenic dogs with dogs having the globular volume and/or platelets scores within reference limits. In this case, if the percentage of anemic and/or thrombocytopenic dogs is greater than that with normal reference values, it is often erroneously concluded that the agent is responsible for these alterations. Moreover, it is also important to evaluate these manifestations within the population of non-reactive dogs. The finding of a ratio that is equal between positive and negative dogs, in this case, would suggest that the presence of the agent had no influence on the observed alterations. In summary, to conclude effectively whether the infectious agent is causing anemia and/or thrombocytopenia in a susceptible dog population, the ratio of these clinical manifestations must be determined in positive and negative animals.

In this study, if only the percentage (28.57%) of anemic dogs that were positive for *E. canis* was analyzed, the results might suggest that this infectious agent is not an important cause of anemia, since more than 70% of these cases could have been attributed to anemia of an unknown origin. However, when the proportion of non-reactive dogs was analyzed, it was demonstrated that the

frequency of anemic dogs is comparatively reduced (13.94%), which therefore indicates that CME was responsible for this alteration.

In conclusion, infection induced by *E. canis* is an important cause of anemia and thrombocytopenia in dogs, while *A. platys* cause primarily only thrombocytopenia. Canine monocytic ehrlichiosis and canine thrombocytic anaplasmosis must be included in the differential diagnoses when anemia and/or thrombocytopenia are observed during routine laboratory evaluations.

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ERRATA

Errata do artigo "Occurrence of *Ehrlichia canis* and *Anaplasma platys* in household dogs from northern Parana" (http://dx.doi.org/10.1590/S1984-29612012005000009), publicado em ahead of print em 04 de dezembro de 2012. No artigo apresenta erro no nome de um dos autores:

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